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PD1 and PDL1 molecules control suppressor activity of regulatory T cells in chronic Chagas cardiomyopathy patients☆

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ABSTRACT

Introduction: Chagas disease, caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*), is the fourth most important tropical disease, which affects approximately 7 million people worldwide. The mechanisms involved in the development of this disease are not completely well understood. An important protective role of regulatory T cells (Treg) in Chagas disease has been observed; however, the specific mechanisms remain unclear. We evaluated apoptosis as a possible mechanism mediated by Treg cells ($CD4^+CD25^{High}FOXP3^+$) to orchestrate the immune response in chronic Chagas disease.

Methods and results: Patients with Chagas disease were grouped as the indeterminate (IND; asymptomatic patients with Chagas disease; n = 10) and dilated cardiomyopathy (CARD; n = 10). Healthy *T. cruzi*-negative individuals (NI; n = 10) were included as a control group. In order to evaluate the apoptotic cell profile, the expression of PD1, PD1L, CD39, CD95, CD95L molecules were investigated. We also evaluated the proportion of $CD14^+$ cells expressing caspase 3. The IND group presented a substantially higher expression of CD39 by Treg cells as compared to the CARD group. On the other hand, the CARD group showed higher expression of PD-1 by Treg cells than both NI and IND groups. Significant positive correlations were observed between Treg $CD95L^+$ cells and CD14 cells expressing caspase 3 as well as between Treg CD39 cells and $CD14^+$ $Caspase3^+$ cells in the IND group.

Conclusion: Our data indicate that the expressions of different molecules that induce apoptosis are associated with suppressive mechanisms mediated by Treg cells and suggest a possible role for PD1 and PDL1 molecules in the morbidity of chronic Chagas disease.

1. Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*), is the fourth most important tropical disease, which affects about 6 to 7 million people worldwide [1]. Although there have been many efforts to control the natural transmission by the triatomine bug, at least 120 million people still are at risk of infection in Latin America and approximately 12 thousand people die annually [2,3]. The disease is characterized by an acute followed by a long and debilitating chronic phase [4]. The acute phase is observed during a short period and is characterized by high parasitemia and intense inflammatory infiltrate. The chronic phase is characterized by a reduced number of parasites in

☆ Written informed consent was obtained from patients for blood samples and this study was carried out in full accordance with all International and Brazilian guidelines and was approved by the Ethics Committee at René Rachou Research Center – FIOCRUZ/MG (14/2006 CEPSH-IRR) and Federal University of Minas Gerais – UFMG (COEP-ETIC 37204).

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the bloodstream and the majority of patients present a clinically silent form of the disease known as the indeterminate form [5]. However, about 20–30% of the *T. cruzi*-infected patients develop a cardiac manifestation of the disease, most commonly dilated cardiomyopathy, which represents the severe form of Chagas heart disease [6].

The host immune response to *T. cruzi* invasion plays an important role in the acute phase of the disease and may be the most important factor determining the severity of the chronic phase [7]. Some parasites modulate the immune response in order to escape and survive. In some cases, the immune modulation is accomplished by alteration of the acquired immune response, including the modification of regulatory T cells (Tregs) phenotype [8]. Regulatory T cells represent 10% of the peripheral $CD4^+$ T cell and maintain immune homeostasis [8]. Regulatory T cells mediate the tone of the immune response and are key players in resolving tissue inflammation and healing [9]. These cells are characterized by expression of the transcription factor, forkhead box P3 (FOXP3) [10]. They suppress inflammation through different mechanisms, which include secretion of inhibitory molecules such as transforming growth factor (TGF)-β, interleukin (IL)-10 and IL-35 and expression of inhibitory molecules such as programmed cell death protein 1 (PD1), cluster differentiation (CD)39 and Fas ligand (CD95L) [11]. PD1 interacts with programmed death-ligand 1 (PDL1) in the target cell and suppresses cytokine production; they also regulate cell survival and proliferation of the target cell [12]. Furthermore, the expression of PD1 by Tregs modulates their homeostasis [13]. Another suppressor mechanism is mediated by the expression of ectonucleotidase, also known as CD39, by Tregs. CD39 hydrolyzes adenosine triphosphate (ATP) to adenosine diphosphate (ADP) or adenosine monophosphate (AMP), with ATP removal limiting the inflammation, and in combination with CD73, with the generation of adenosine which inhibits the immune response [14]. Regulatory T cells also mediate the immune response through the expression of CD95L, which interacts with CD95 and causes the apoptosis of the target cell [15].

During the chronic phase of Chagas disease, IND patients present a higher frequency of Tregs than CARD patients and its frequency is correlated with a better cardiac function on IND patients. Regulatory T cells from IND patients produce IL-7, IL-10, granzyme B with Treg frequency correlating with the frequency of $AnnexinV^+$ CD4 T cells. On the other hand, Tregs from CARD patients express higher levels of CTLA4 than Tregs from IND patients [16]. Although several studies have investigated the role of Tregs and its suppressor mechanisms in Chagas disease its function has not yet been clearly determined. In the present study, we showed that Tregs from IND patients express higher levels of CD39 and CD95L when compared to CARD patients. Furthermore, we observed a positive correlation in the expression of PD1 and PD1L by Tregs in the CARD group, indicating different suppressive mechanisms involved with the development of distinct forms of Chagas disease.

2. Materials and methods

2.1. Study population

Patients were identified and selected at the Referral Outpatient Center for Chagas Disease of the Hospital das Clínicas of *Universidade Federal de Minas Gerais* (UFMG), Brazil. Serology for *T.* cruzi was determined by two or more tests (indirect immunofluorescence, ELISA, indirect haemagglutination) and patients were considered as infected when at least two different tests were positive. This study was approved by the Ethics Committee of the Instituto Rene Rachou- FIOCRUZ (14/2006 CEPSH-IRR) and by UFMG (COEP-ETIC 37204). Written informed consent was obtained from all individuals prior to their inclusion in the study. Independent of their participation in this study, all individuals enrolled were submitted to a standard screening protocol, follow up and clinical treatment. We excluded patients with systemic arterial hypertension, diabetes mellitus, thyroid dysfunction, renal disease, chronic obstructive pulmonary disease, electrolytic disorders, alcoholism, and clinical history suggestive of coronary artery obstruction or rheumatic disease, as well as the impossibility of undergoing the standard examinations.

Patients with chronic Chagas disease were grouped as indeterminate (IND; asymptomatic patients with Chagas disease) and dilated cardiomyopathy (CARD) as previously reported [16]. The IND group included 10 patients ranging in age from 30 to 70 years, with no significant alterations in electrocardiography, chest X-ray, echocardiogram, esophagogram, and barium enema. The CARD group included 10 patients with age ranging from 30 to 70 years, with evidence of right and/or left ventricular dilation, global left ventricular dysfunction, and alterations in the cardiac electric impulse generation and conduction. In this group, the cardiac dysfunction was evidenced by electrocardiograms, chest X-rays, and echocardiography, which showed the occurrence of heart enlargement in all CARD patients analyzed. Left ventricular ejection fraction (LVEF) and left ventricular diastolic diameter (LVDd) were used as clinical parameters of left ventricular function for patients with Chagas disease enrolled in the present study [16,5] Healthy individuals (N = 10), ranging in age from 29 to 55 years, from a non-endemic area for Chagas disease and showing negative serological tests for *T. cruzi* were included as a control group (NI).

2.2. Trypanosoma cruzi soluble antigen preparations (TRYPO)

Trypanosoma cruzi parasites from the Y strain were grown in L-929 cell line as described in a previous study [17]. Cells were obtained by inoculation of 10 trypomastigotes forms per cell and free trypomastigostes were removed through washing with culture medium. Then, cells containing trypomastigotes were maintained in RPMI medium (Sigma Aldrich) enriched with 5% fetal calf serum and antibiotic (Penicillin, 500 U/mL and Streptomycin 0.5 mg/mL (Sigma Aldrich) for approximately 5 days. After this period, trypomastigotes were collected from the supernatant. Contamination with amastigotes were always lower than 3%. The trypomastigotes collected from the supernatant were used to obtain the *T. cruzi* antigens. Antigen preparation was performed as described by MORATO et al, 1986 [18]. The protein quantified by Bradford method. *T. cruzi* soluble antigens were stored at −70 °C and final concentration of 20 μg/mL of the antigen used.

2.3. Flow cytometric analysis of cells culture

Whole blood was stimulated with either medium alone (RPMI 1640 supplemented with 1.6% L-glutamine, 3% antibiotic-antimycotic, 5% of AB Rh-positive heat-inactivated normal human serum) or Trypo (20 μg/mL) for 22 h at 37 °C and 5% CO_2 [16]. During the last 4 h of culture, Brefeldin A (Sigma, St. Louis, MO, USA) (10 μg/mL) was added to the cultures [19]. Cultured cells were washed twice in PBS containing 1% bovine serum albumin and stained with CD4 (peridinin chlorophyll-*a* protein linked to cyanine 5.5-PERCP-Cy5.5) (SK3), CD25 (V450) (M-A251), CD39 (phycoerythrin-PE) (TU66), CD95 (Fas) (fluorescein isothiocyanate-FITC) (DX2) and CD178 (FasL) (PE) (NOK-1), CD274 (PDL1) (PE-Cy7) (MIH1) (PE), CD279 (PD1) (FITC) (MIH4) (PE) monoclonal antibodies all purchased in BD Pharmingen (USA). The cells were then fixed in formaldehyde (4%) and permeabilized in saponin buffer (0.5%) (Sigma, USA) for 15 min. Then, the cells were incubated with monoclonal antibody Foxp3 (APC) (236A/E7) (E-Bioscience, USA).

In parallel, apoptosis of CD14 cells was evaluated through the staining of caspase 3. For this study, aliquots of 150 μL of blood were transferred to polystyrene tubes and incubated for 30 min at room temperature with 2 μL of anti-CD14 (MϕP9) (PERCP) (BD Pharmigen, USA). The tubes were incubated in the dark for 30 min at room temperature. The cells were permeabilized with saponin buffer (0.5%) (Sigma) for 15 min at room temperature in the dark. Finally, the cells were incubated with PE anti-active caspase 3 mAb (C92-605) (BD

Pharmingem, USA) using 2 μL/1 × 10^6 cells for 60 min at room temperature in the dark.

Phenotypic analyses were performed by flow cytometry with a Becton Dickinson FACSCANTO II flow cytometer, collecting data on 5 × 10^4 lymphocytes (gate by forward and side scatter properties) and analyzed using FlowJo software. The phenotypic profile of Tregs and their molecules analyses was determined as previously described by De Araujo et al., 2012 [16].

2.4. Statistical analysis

Analyses were performed using GraphPad Prism software, version 5.0. The following nonparametric tests were performed: 1) Mann-Whitney test for pairwise comparisons between the groups and 2)

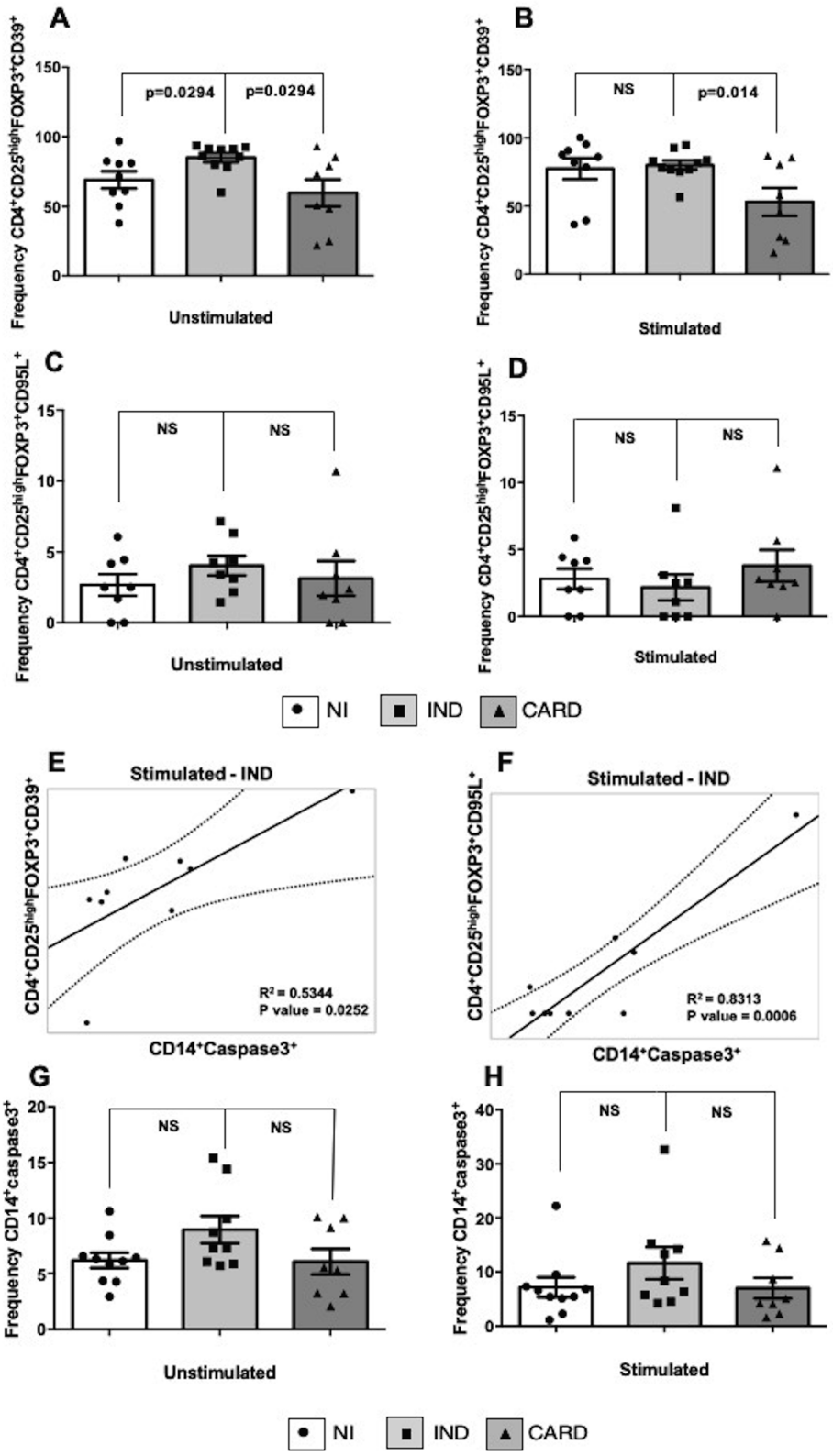

Fig. 1. $CD4^+CD25^{High}FOXP3^+$ T cells from IND patients express higher levels of CD39 compared to CARD patients and positive correlation between $CD39^+$ Treg and $CD14^+$caspase3^+ as well as $CD95L^+$ Treg and $CD14^+$caspase3^+. Subjects were grouped as IND (N = 10) and CARD (N = 10) patients or NI (N = 10) healthy controls. Panel A. Proportion (%) of unstimulated $CD4^+CD25^{High}FOXP3^+CD39^+$ T cells. Panel B. Proportion (%) of $CD4^+CD25^{High}FOXP3^+CD39^+$ T cells after in vitro stimulation with *T. cruzi* antigens. Panel C. Proportion (%) of unstimulated $CD4^+CD25^{High}FOXP3^+CD95L^+$ T cells. Panel D. Proportion (%) of $CD4^+CD25^{High}FOXP3^+CD95L^+$ T cells after in vitro stimulation with *T. cruzi* antigens. Panel E. Correlation between $CD4^+CD25^{High}FOXP3^+CD39^+$ T cells and $CD14^+$caspase3^+ cells. Panel F. Correlation between $CD4^+CD25^{High}FOXP3^+CD95L^+$ T cells and $CD14^+$ caspase 3^+ cells from IND patients after in vitro stimulation with *T. cruzi* antigens. Panel G. Proportion (%) of unstimulated $CD14^+$caspase3^+ cells in NI, IND and CARD groups. Panel H. Proportion (%) of stimulated $CD14^+$caspase3^+ cells in the different groups.

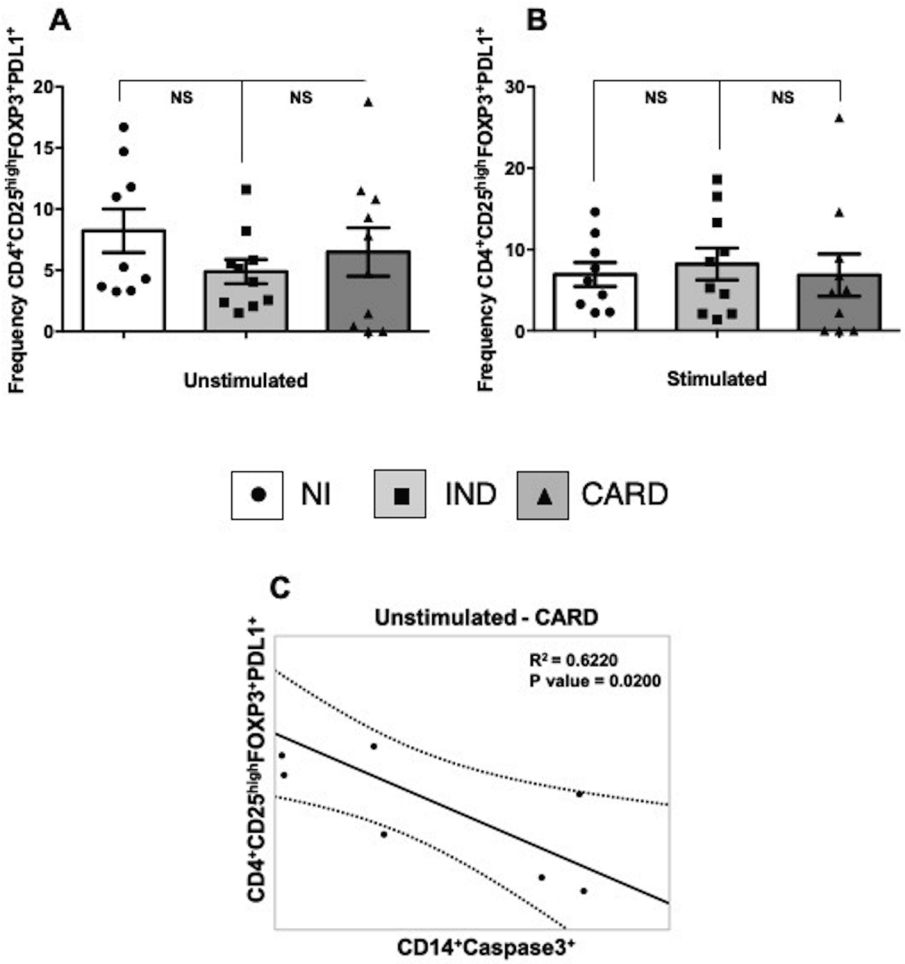

Fig. 2. PD1L expression on $CD4^+CD25^{High}FOXP3^+$ cells does not differ among the groups and it does not correlate with the proportion of $CD14^+Caspase3^+$ cells. Profile of $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells from patients with Chagas disease. Subjects were grouped as IND (N = 10) and CARD (N = 10) patients or NI (N = 10) healthy controls. Panel A. Proportion (%) of unstimulated $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells. Panel B. Proportion (%) of $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells after in vitro stimulation with *T. cruzi* antigens. Panel C. Correlation between the proportion of unstimulated $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells and $CD14^+$caspase3^+ cells from patients from CARD group.

Kruskal-Wallis test for multiple comparisons among the three groups. Pearson correlation coefficients were assessed, and analyses conducted with *JMP 5.0.1* software from *SAS*. For all inferential analyses two-sided p-values are reported and p-values < 0.05 were considered statistically significant.

3. Results

3.1. CD39 and CD95L expression by $CD4^+CD25^{High}FOXP3^+$ T cells is correlated with the apoptosis of monocytes in patients with the indeterminate form of Chagas disease

The evaluation of CD39 expression by $CD4^+CD25^{High}FOXP3^+$ T cells showed significantly higher levels of this molecule in the IND group when compared to NI and CARD groups both in the ex vivo and culture (Fig. 1A). After in vitro stimulation with *T. cruzi* antigen, $CD4^+CD25^{High}FOXP3^+$ T cells from CARD patients expressed lower levels of CD39 compared to IND patients (Fig. 1B).

No significant difference was observed in the expression of CD95L by unstimulated $CD4^+CD25^{High}FOXP3^+$ T cells or Trypo stimulated $CD4^+CD25^{High}FOXP3^+$ T cells (Fig. 1C, 1D). In addition, there was a positive correlation between the frequency of $CD4^+CD25^{High}FOXP3^+CD39^+$ T cells and $CD14^+Caspase3^+$ (Fig. 1E) and between the frequency of $CD4^+CD25^{High}FOXP3^+CD95L^+$T cells and $CD14^+$ caspase3^+ cells (Fig. 1F). No difference was observed in the percentage of $CD14^+$ caspase3^+ cells comparing the different groups in the distinct conditions unstimulated or stimulated with T. cruzi antigen (Fig. 1G and H).

3.2. The expression of PD1L by $CD4^+CD25^{High}FOXP3^+$ T cells is negatively correlated with apoptosis of monocytes in cardiac patients

Another mechanism used by Treg cells to suppress the immune response is the expression of PD1L. No significant difference was observed in the expression of PD1L by unstimulated or TRYPO stimulated $CD4^+CD25^{High}FOXP3^+$ T cells of IND patients compared to CARD patients (Fig. 2A, 2B). Considering the unstimulated culture, the data showed a negative correlation between the proportion of $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells and $CD14^+$ Caspase3^+ in CARD patients (Fig. 2C), which indicates that the expression of PD1-L by Tregs is not involved with the apoptosis of monocytes.

3.3. $CD4^+CD25^{High}FOXP3^+$ T cells may use PD1 and PD1L expression as a suicidal mechanism in Chagas disease patients with dilated cardiomyopathy

Since PD1 expression indicates cell exhaustion and its expression is related to apoptosis through interaction with PD1L, we focused on the expression of this molecule as well as its correlation with PD1L expression. The CARD group showed a higher proportion of unstimulated $CD4^+CD25^{High}FOXP3^+PD1^+$ T cells when compared to control group (Fig. 3A). No significant difference was observed in the expression of $CD4^+CD25^{High}FOXP3^+PD1^+$ T cells after in vitro stimulation with *T. cruzi* antigens (Fig. 3B). In contrast, a significant positive correlation between the expression of PD1 by $CD4^+CD25^{High}FOXP3^+$ T cells and the expression of PD1L by these cells at unstimulated culture was observed in the CARD group (Fig. 3C). These findings indicate that PD1 and PD1L could be involved in the mechanism associated with the

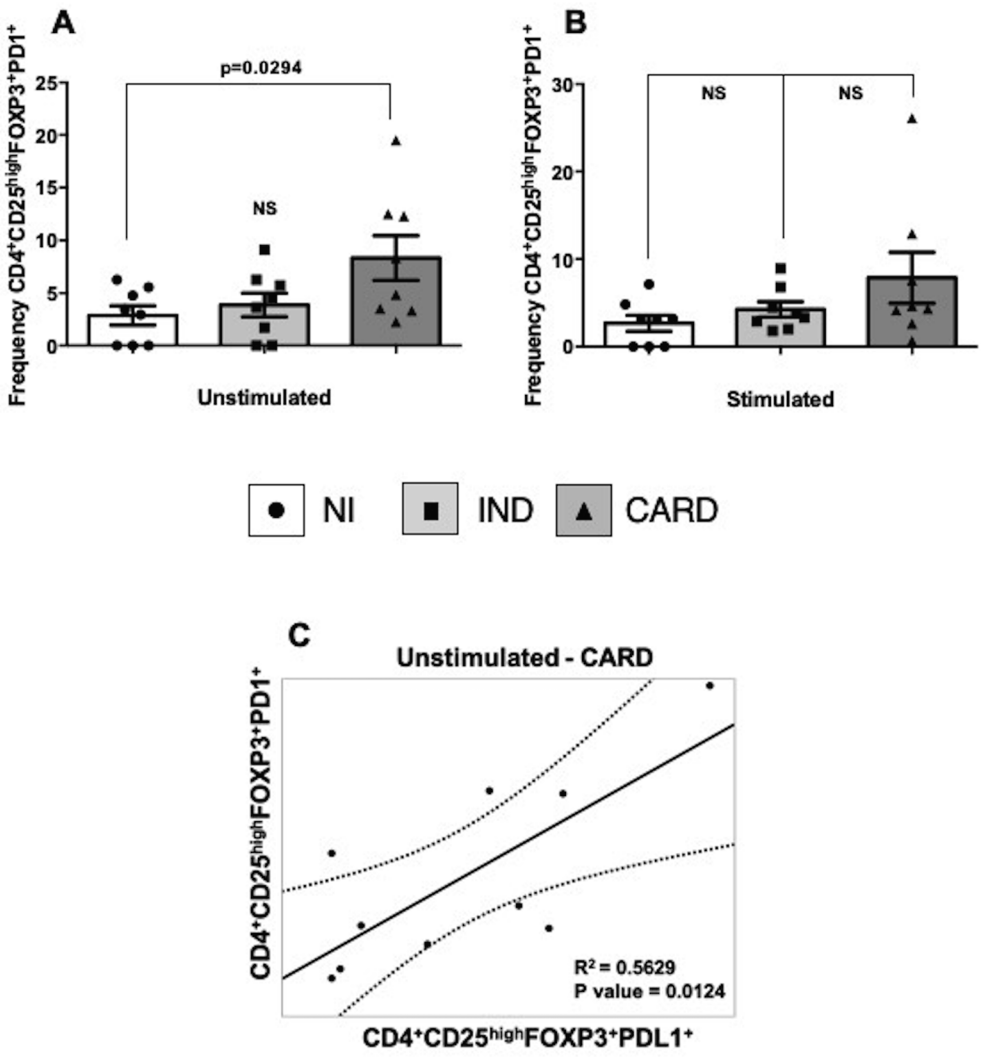

Fig. 3. PD1 expression was higher in $CD4^+CD25^{High}FOXP3^+$ cells from the CARD group compared to the control group and positive correlation was observed between the proportion of $CD4^+CD25^{High}FOXP3^+PD1$ and the proportion of $CD4^+CD25^{High}FOXP3^+PD\text{-}1L^+$ T cells. Subjects were grouped as IND (N = 10) and CARD (N = 10) patients or NI (N = 10) healthy controls. Panel A. Proportion (%) of unstimulated $CD4^+CD25^{High}FOXP3^+PD\text{-}1^+$ T cells. Panel B. Proportion (%) of $CD4^+CD25^{High}FOXP3^+PD\text{-}1^+$ T cells after stimulation with *T. cruzi* antigens. Panel C. Correlation between unstimulated $CD4^+CD25^{High}FOXP3^+PD1^+$ T cells and $CD4^+CD25^{High}FOXP3^+PD1L^+$ T cells from CARD patients.

lower proportion of Tregs observed in CARD patients.

4. Discussion

In this present study, we showed that Tregs of IND patients express higher levels of CD39 and CD95L, whereas, in CARD patients, a positive correlation was observed in the expression of PD1 and PD1L in Tregs, indicating distinct suppressive mechanisms involved with the different forms of Chagas disease.

Several factors have been described to be involved with the development of different forms of Chagas disease. *T. cruzi* induces innate and adaptive immune responses and that the immune response against several parasites is regulated through suppressive mechanisms [20]. The role of Treg cells has been observed in this same context [21]. However, the mechanisms used by Treg cells ($CD4^+CD25^{High}FOXP3^+$) to suppress the immune system are not well comprehended [22].

Previous data from our group showed that IND patients present a higher proportion of $CD4^+CD25^{high}$ $FOXP3^+$ T cells when compared to CARD patients or healthy *T. cruzi*-negative individuals, indicating an important role of Treg cells in the control of the host immune response induced by the parasite and in the pathogenesis of chronic Chagas cardiomyopathy [16].

The higher expression of CD39 by $CD4^+CD25^{High}FOXP3^+$ T cells as well as the positive correlation between the frequency of $CD4^+CD25^{High}FOXP3^+CD39^+$ T cells and $CD14^+$ caspase 3^+ from IND group provided evidence of the importance of this molecule in the immune response in Chagas disease. This finding suggests that the expression of CD39 could be a mechanism involved with the control of the inflammatory response in IND patients. Previous studies have shown a relationship between the expression of CD39 and suppression of the immune response in several pathologies [23]. In the context of Chagas disease, Santos et al. (2009) [24] demonstrated that one of the possible mechanisms by which *T. cruzi* escapes immune attack is by the expression of enzymes called ectonucleotidases (CD39 family) that would be responsible for its infectivity and virulence and at the same time cleave ATP and reduce the inflammatory response. These findings provided evidence of the association of suppressive mechanism involved with the expression of CD39 in chronic Chagas disease leading the host adaptation to the parasite and preventing the disease progression [25].

Our data also showed higher expression of PD-1 by Treg cells in the CARD group compared to the NI group. Furthermore, there was a positive correlation between the expression of PD-1 and PD-1L molecules expressed by $CD4^+CD25^{High}FOXP3^+$ T cells in the CARD patients, suggesting that Treg cells from CARD patients may be dying via apoptosis due to the interaction between PD-1L and its receptor PD-1, both expressed by $CD4^+CD25^{High}FOXP3^+$ T cells. On this note, our data suggest that apoptosis of Treg cells might occur in CARD patients reducing the suppression of target cells. Furthermore, the apoptosis of $CD4^+CD25^{High}FOXP3^+$ T cells in CARD patients corroborates the lower frequency of these cells when compared to IND patients [16].

In contrast to findings from a previous study [26], here, we

observed a positive correlation between the frequency of $CD4^+CD25^{High}FOXP3^+CD95L^+$ T cells and the apoptosis of monocytes in IND patients. This finding indicates that in asymptomatic Chagas disease patients, Treg cells might be associated preferentially with the induction of apoptosis of monocytes. Thus, the expression of both CD95L and CD39 molecules could be associated with the regulated inflammatory response presented by IND patients.

Prior kinetics studies from our group to evaluate the expression of FOXP3 by $CD4^+CD25^{high}$ T cells have demonstrated that Treg cells from IND patients maintain the FOXP3 expression after in vitro stimulation with *T. cruzi* antigens, whereas Treg cells from CARD patients exhibited reduced expression of FOXP3 after the third day of cell culture [16]. This finding also corroborates with the hypothesis that possible apoptosis of Treg cells observed in CARD patients might be through a suicidal mechanism mediated by the expression of PD-1 and PD-1L.

Our findings indicate that the expressions of different molecules that induce apoptosis are associated with suppressive mechanisms mediated by Treg cells and suggest a possible role for PD1 and PDL1 molecules in the morbidity of chronic Chagas disease.

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Competing interests

The authors declare that are no competing interests.

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